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# **Product diversity linked to substrate usage in chain elongation by mixed culture fermentation**

*Running title:* **Microbial versatility for chain elongation**

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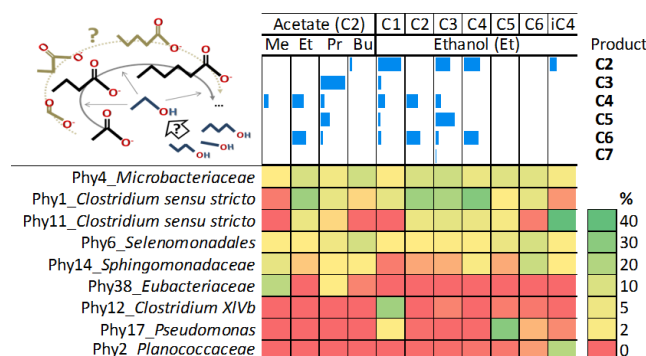
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# TOC Art



## Abstract

Acetate and ethanol can be converted to caproic acid by microorganisms through reverse  $\beta$ -oxidation. There is limited insight into the versatility of chain elongation in view of different starting substrates, including even and odd carbon carboxylates and alcohols other than ethanol. Thermodynamic analyses show that most elongation pathways are energetically feasible. Through incubations of microbial communities with different substrate-pair combinations, we established that ethanol and propanol were both highly suitable for chain elongation. As electron acceptor, acetate, propionate and butyrate readily elongated with ethanol whereas an adaptation period was necessary for formate. Isobutyrate and longer chained fatty acids above butyrate were not elongated. The microbial communities converged and consistent enrichment of *Clostridium* spp. was observed, independent of the supplied alcohol or carboxylate, with a strain related to *Clostridium kluyveri* dominating the enrichments. Community analysis also showed phylotypes related to *Bacteroidaceae* and *Microbacteriaceae* families in all tests, which are capable of converting the base substrates to useful intermediates. These organisms were mainly enriched with methanol or formate. Our overall conclusion is thus that multiple substrates can be used for chain elongation, and that this process is carried out by highly similar organisms for direct chain elongation, irrespective of the substrate.

**Keywords:** alcohols; fermentation; medium chain fatty acids; mixed culture; reverse- $\beta$ -oxidation.

## INTRODUCTION

Side streams with considerable organic content can be used as inputs for production of chemicals such as carboxylic acids. For this, mixed microbial cultures can be used as they hydrolyze and ferment the residual organics from these low value feedstock to e.g. short chain fatty acids (SCFAs). This process is known as the carboxylate platform. Carboxylates can be concentrated in the fermentation broth or undergo secondary (bio)conversion to facilitate product recovery through production of biopolymers, electrosynthesis or chain elongation to medium chain fatty acids (MCFAs) <sup>1</sup>. A key bottleneck in the carboxylate platform is the heterogeneity of the feedstock, as they tend to lead to bioconversion to non-specific products at moderate to low titers which makes downstream processing difficult. Modular cascade processes, in which each step focuses on the exploitation of a selected organic fraction, may lead to higher efficiencies<sup>2-4</sup>.

A proportion of microorganisms within mixed communities are able to perform reverse  $\beta$ -oxidation in which the fermentation intermediates, SCFAs, are elongated to MCFA that attract higher value and are easier to separate <sup>5</sup>. Energy-rich, reduced compounds are required to provide energy, reducing equivalents and acetyl-CoA so that this pathway can proceed <sup>6</sup>.

The synthesis begins with the condensation of acetyl-CoA which is then further reduced with the addition of two carbons through each subsequent round of fatty acid synthesis.

Consequently, acetate (C2) can be elongated to butyrate (C4) and further to caproate (C6) and caprylate (C8). Odd carbon chains can also be produced when cellular propionyl-CoA levels are high, because of their incorporation in place of acetyl-CoA in the initial step of fatty acid synthesis. In this case, propionate (C3) will be elongated to valerate (C5) and further to heptanoate (C7)<sup>7</sup>.

*Clostridium kluyveri* is of the most studied organism performing chain elongation. It can grow on ethanol, the electron donor, and a SCFA salt, but is unable to attack substrates like glucose which are commonly fermented by other anaerobic bacteria <sup>8</sup>. *C. kluyveri* was also found to be able to grow on propanol <sup>9</sup> but the main fermentation product was propionate <sup>10</sup>. Thus, the role of propanol as an electron donor for chain elongation is uncertain. Other isolated organisms have been described to perform chain elongation, but as recently reviewed in the literature, their activities rely on the conversion of simple substrates, mainly short chain carboxylates. Some specific organisms may grow on monomeric sugars, amino acids and carbon dioxide, with energy conservation through pyruvate <sup>11</sup>. The fact that they are unable to degrade complex substrates, composed of lipids, carbohydrates and proteins, makes it necessary to use co-cultures or mixed cultures. Other hydrolytic or fermentative bacteria within a mixed culture will degrade complex substrates to a mixture of typical intermediates suitable for chain elongation.

The titers of MCFA tend to be rather low (Table S1, supporting information), mostly due to the toxicity they exert under acidic conditions. Higher concentrations can be achieved at neutral pH, however in this case methanogenesis may occur and consume SCFA. To deal with these issues, chemical inhibitors to avoid methane production or in-line extraction systems, such as liquid-liquid extraction (pertraction) systems <sup>12</sup>, are required to decrease the product concentration and alleviate the toxicity issue. Other conditions that can promote chain elongation are mesophilic temperatures (30-37°C), a low CO<sub>2</sub> concentration and the presence of SCFA instead of lignocellulosic material (references as reported in Table S1).

Besides ethanol and acetate, substrates such as propanol (co-generated with bioethanol) or odd carbon sugars such as pentoses (e.g. xyloses) or heptoses (e.g. mannoheptulose) fermentable to odd carbon SCFA or alcohols can be present in biorefinery side-streams.

Combinations of substrates with even number of carbons (from now on even substrates, e.g.

C2 or C4) and odd number of carbons (from now on odd carbon substrates, e.g. C3) produce mixtures of short and medium chain carboxylates, while combinations of both odd carbon substrates (e.g. propionate and propanol) were not reported thus far.

Overall, there is a limited understanding of the metabolic potential of mixed populations to deal with various input alcohols and carboxylates, what products will be formed and how microbial populations will correlate with different feedstock. It is still unclear if the substrate is the driving force for microbial community enrichment, i.e. different substrates will lead to different communities. Therefore, we evaluated thermodynamic considerations based on the output product and yields of different carboxylate-alcohol substrate-pair combinations, including even and odd carbon substrates, applying the same operational conditions to the same initial mixed culture. 16S rRNA gene sequencing was used to track changes in the composition of communities enriched under different substrate combinations for chain elongation, using the same starting community.

## **MATERIALS AND METHODS**

### **Mixed culture inoculum**

A concentrated mixed culture microbiome was obtained by centrifugation (300 s, 14000 g) of the effluent collected from a chain elongation fermenter fed with acetate and ethanol at a molar ratio of 1:3. The supernatant was discarded to avoid substrate interferences and pellets were resuspended with a fixed volume of RO water. The fermenter was run for three months at 35 °C as a continuous stirred tank reactor (CSTR) and consisted of a water-jacketed glass cylinder with a working volume of 2.4 L operated with an HRT of 10 days at pH  $5.5 \pm 0.2$ . The initial sludge inoculum was obtained from a full-scale anaerobic digester treating potato

waste. When samples were collected from the effluent of the chain elongation reactor, butyrate was the main product outcome.

## **Experimental procedure**

MCFA production was stimulated using different combinations of carboxylates or alcohols with acetate or ethanol as co-substrate, respectively, in molar ratios of 1:2. This ratio was chosen to ensure initial alcohol oxidation as described in Spirito et al. <sup>6</sup>. Carboxylate sets were fed with 50 mM of formate, acetate, propionate, butyrate, iso-butyrate, valerate or caproate and 100 mM of ethanol. Alcohol sets were fed with 100 mM of methanol, ethanol, propanol or butanol and 50 mM of acetate. The experimental set-up and medium composition is defined in supporting information.

A total of four runs of batch tests in triplicates were performed. The inoculum for all tests was obtained from the same reactor effluent for all tests. A first run with low biomass (Run 1-LB, 54 mg VSS L<sup>-1</sup>, Pielou evenness value of  $0.73 \pm 0.01$ ; Richness value of  $295 \pm 19$ ) was carried out for 9 days. This low biomass obtained in the first run is unrealistic for bioreactors. Therefore, to disregard substrate inhibitions to biomass a more concentrated inoculum was used for a second run at higher biomass concentration (Run 1-HB; 271 mg VSS L<sup>-1</sup>, Pielou evenness value of  $0.66 \pm 0.01$ ; Richness value of  $255 \pm 21$ ). For the high biomass test, three consecutive enrichments were performed. For DNA analysis biomass was sampled from the inoculum of both low (n = 3) and high (n = 2) biomass and at the end of each triplicate from Run 1-LB, Run 1-HB and Run 3-HB. Chemical and molecular analysis performed during the experiments are detailed in Supporting Information (SI).

## RESULTS AND DISCUSSION

### Thermodynamics of chain elongation favor all carboxylate-alcohol combinations

In anaerobic fermentations, metabolic reactions take place close to thermodynamic equilibrium with minimum energy dissipation; consequently, changes in product and substrate concentrations can easily reverse the driving force of the (bio)chemical reaction<sup>13,14</sup>. To understand the interactions during anaerobic fermentation, thermodynamic calculations of different reactions were performed based on standard Gibbs energy ( $\Delta G^0$ ) obtained from the formation values of individual compounds and corrected for the working temperature ( $\Delta G^0_{35^\circ\text{C}}$ ) and biochemical conditions ( $\Delta G^{01}_{35^\circ\text{C}}$ ) (Table 1). Both higher temperature and biochemical conditions decreased the energy required for reactions to occur compared with standard conditions ( $\Delta G^0 > \Delta G^{01}_{35^\circ\text{C}} \geq 0$ ) or increased the energy gain in some reactions ( $\Delta G^{01}_{35^\circ\text{C}} < \Delta G^0 < 0$ ). Alcohols oxidation should not take place according to unfavorable thermodynamic values (Eq. 1 to 4). However, the energy requirement under standard biological conditions is close to equilibrium ( $\Delta G^{01}_{35^\circ\text{C}} \approx 0 \text{ KJ mol}^{-1}$ ) and thus small modifications on the environment (e.g. pH or substrate concentrations) would promote the oxidation of alcohols to carboxylic acids and hydrogen.

Table 1 (containing references<sup>15,16,17</sup> and<sup>18</sup>)

Carboxylates as a single source may not be oxidized with the exception of formate (Eq. 6). Most of the VFA oxidation reactions associated with hydrogen production present high-energy requirements whereas at least some of these reactions occur naturally in bioprocesses (Eq. 11, 20, 26)<sup>19</sup>. For such processes, the hydrogen partial pressure ( $P_{\text{H}_2}$ ) affects the energy requirements, for example low  $P_{\text{H}_2}$  enables the complete pathway of acetate oxidation to  $\text{CO}_2$  and  $\text{H}_2$ <sup>14</sup> whereas high  $P_{\text{H}_2}$  favors reductive outcomes as in fermentation.



Overall, chain elongation reactions represent favorable thermodynamics for all carboxylate – alcohol combinations and with a higher energy gain when hydrogen is present instead of alcohols (Table 1).. Conversion of acetate to butyrate and caproate is thermodynamically possible in the presence of hydrogen (Eq. 13 & 14), although acetate conversion directly to caproate would require higher  $P_{H_2}$  as indicated by stoichiometry. Chain elongation is also plausible through auto-condensation of butyrate although the low energy gain ( $\Delta G^{01}_{35^\circ C} = -0.2$  KJ mol<sup>-1</sup>, Eq. 28) would direct the substrate to more energetically beneficial reactions. Contrarily, propionate has a broader reaction competition. While propionate elongation to valerate in the presence of ethanol is thermodynamically feasible and close to the equilibrium (Eq. 25), the conversion to propanol (Eq. 21) or the re-oxidation to acetate, CO<sub>2</sub> and H<sub>2</sub> (Eq. 24) could only be possible after environmental changes.

Under equilibrium conditions the most energetic pathway would dictate the outcome product, however it must be taken into account that Gibbs energy is dependent on the concentration of each compound ( $\Delta G = \Delta G^0 + RT \ln Q$ ). Therefore, only reactions without the presence of hydrogen and carbon dioxide, even with a  $\Delta G^0 > 0$ , would be possible during the start-up period where there is no product concentration, while chain elongation reactions such as Eq. 5, 14, 22 or 29 will be favored as soon as H<sub>2</sub> and CO<sub>2</sub> start to accumulate.

### **Chain elongation with different alcohols**

In a first series of batch tests, we compared the consumption of different alcohols (C1 to C4) with acetate, both at low and high biomass concentrations (Run 1- LB; Run 1-HB; Figure 1). pH was maintained at the end of the tests with LB while it increased up to  $0.45 \pm 0.05$  units with HB. With LB the net methanol, ethanol and propanol consumption was higher compared to HB (50 mM higher for methanol and 10 mM for ethanol and propanol), likely due to additional alcohol formation from the excess inoculum. This is evidenced by the higher

acetate consumption with HB leading to a higher production of elongated VFAs. The cell density is thus an important parameter to consider for mixed culture batch tests, a fact generally not considered, as too much substrate per cell unit might inhibit the final concentration in the broth. Butanol consumption and its VFA production was negligible in comparison with the other alcohols. Butanol is presumably toxic to for example *Clostridium acetobutylicum* and *C. beijerinckii* that produce it<sup>20</sup>. Although the starting alcohol concentration (100 mM) was under the maximum inhibition found during its production (13 g L<sup>-1</sup>; 176 mM), non-adapted communities may have a higher sensitivity to butanol.

Net ethanol production was observed in all tests including the control while not added as a substrate. This suggested that reaction 12 (acetate reduction; Table 1) was occurring simultaneously. If ethanol was mainly produced by cell lysis, ethanol concentrations in the HB control test would be significantly different from the low biomass test, which was not the case (Figure 1a). Therefore, auto-formation of ethanol together with acetate allowed chain elongation to even carboxylates (C4 and C6) in all conditions tested (Figure 1b). Further chain elongation of butyrate to caproate was observed when ethanol was supplied in batch tests to a maximum of 24.5 mM (2.8 g L<sup>-1</sup>). When propanol was provided as electron donor, degradation of this alcohol to propionate (Eq. 3) was the main process in LB, but chain elongation to valerate (Eq. 18) increased from 11 to 27% of the total molar VFA production (Table S2, supporting information) when the biomass concentration was increased. Valerate titers achieved in the HB test (16 mM; 1.63 g L<sup>-1</sup>) were comparable to those obtained from targeted engineered organisms (2.58 g L<sup>-1</sup>)<sup>21</sup>. No methane production was detected in any of the tests performed. Hydrogen was only detected in HB using ethanol (23.8 mM) and propanol (6.8 mM) corroborating the hypothesis that fermentation of excess inoculum produced hydrogen instead of consuming it. Concluding, with our inoculum, ethanol and propanol were the only possible electron donors for chain elongation of acetate with total

VFA productions of 10.8 and 7.8 g COD L<sup>-1</sup>, respectively, while any carboxylate production under other conditions was due to substrate degradation.

### **Enrichment with different alcohols for VFA production**

Transfers of the microorganisms to new substrate with different alcohols were performed to investigate adaptation and to eliminate the cell lysis effect. Whereas pH increased in the first HB run, it decreased to a maximum of 0.6 units ( $0.2 \pm 0.2$  units as average) in the last enrichment. Net carboxylate production decreased with all alcohols, except propanol, throughout transfers (Run 3-HB; Figure S1a, supporting information). Ethanol was not further detected within the different transfers when using methanol, propanol or butanol, and showed no productions due to lysis. Neither alcohols nor VFAs were detected in the control test after the third transfer (data not shown). The initial overshoot of chain elongation to butyrate and further to caproate was stabilized at lower concentrations after two consecutive transfers due to a decrease of substrate/inoculum degradation to ethanol. The final VFA composition (Table S2) for methanol tests was mainly butyrate (97%) while proportions in the acetate-ethanol conditions were maintained over the enrichment (54% butyrate and 46% caproate). The fact that with methanol (C1) only carboxylates with even carbon numbers were produced was attributed to an oxidation to formate (Eq. 1), and formate further elongated to acetate or ethanol (Eq. 7 and 8) in the presence of hydrogen (maximum detected of 0.8 mM) or due to side reactions with acetate (Eq. 12 and 16). Thus, methanol was not used to directly elongate carboxylates. Contrarily, propanol degradation increased with new transfers leading to an increase of odd carbon carboxylates such as propionate and valerate in the final composition (Table S2). Concluding, alcohols such as methanol, ethanol and propanol, but not butanol, are suitable for producing MCFA, and ethanol and propanol are the most successful. Combinations of acetate-propanol lead to a mixture of even and odd carbon carboxylate mixtures, while the rest, including methanol, lead to a mixture of even carboxylates.

## Chain elongation with different carboxylates as substrate

Different carboxylates from C1 to C6 including isobutyrate were tested for chain elongation using ethanol as electron donor with low and high biomass concentrations (LB, HB). pH varied only within  $0.12 \pm 0.04$  units at the end of LB tests while it increased by an average of  $0.44 \pm 0.07$  units in HB tests. Acetate and ethanol were generated with LB and HB (Figure 2) when formate (C1) was used as a substrate, thus combining Eq. 6, 7, 8 and 12. The total ethanol available included that generated *in situ* and the ethanol fed as substrate, giving a net consumption of zero at the end of the test. In Run 1-HB propionate, butyrate and some traces of caproate (0.46 mM) were detected, together with considerable hydrogen production (equivalent to 41.50 mM in 9 days). The hydrogen production was likely due to the aforementioned inoculum decay as corroborated by its presence within the formate control tests (Figure S2, supporting information). Although the final VFA composition varied from LB to HB with formate (Table S3), probably due to hydrogen influence (Eq. 7 and 8 compared to 9 without H<sub>2</sub>), no significant increase in total carboxylate production was observed by increasing biomass concentration (6.2 mM *versus* 6.7 mM). Formate was solely degraded to CO<sub>2</sub> and H<sub>2</sub> when ethanol was not present as a substrate in the control tests (Eq. 6; Figure S2).

Tests with acetate or propionate with ethanol as substrates performed as expected according to literature (Table S1), generating a maximum of 10.8 g COD L<sup>-1</sup> of carboxylates. Consumption of the corresponding substrates and productions of VFAs and hydrogen rose when increasing the biomass concentration. Acetate was first elongated to butyrate and the latter to caproate either with ethanol or hydrogen (with CO<sub>2</sub> for carbon addition) as electron donors (Eq. 29 to 31). Hydrogen production in the system increased from 1.8 mM with LB to 23.8 mM with HB. The same behavior was observed in the control test without ethanol addition, probably by ethanol generated through inoculum decay and subsequent acetate

reduction (Figure S2). In the case of propionate, some conversion of the substrate to acetate and to propanol (3.7 mM and 5.6 mM with LB and HB, respectively) was observed (also for the control test, Figure S2). The main elongated product from the propionate-ethanol mixture was valerate (30.71 mM or 3.1 g L<sup>-1</sup>; 52 - 54%, Table S3, supporting information), with traces of heptanoate emerging by further valerate elongation (Eq. 35, 0.8 mM with LB, 3.5 mM with HB). Heptanoate production could be targeted in contrast with other substrates as the propionate-ethanol combination was the only one elongating to C7 (Figure 2b, Table S3).

Under a butyrate-ethanol combination the main products detected were acetate and caproate. With LB the oxidation of either butyrate or ethanol (Eq 26 and 2, respectively) occurred. Chain elongation was enhanced with HB with a final composition of 90% caproate (Table S3) which accounted for 4.9 g L<sup>-1</sup>. This final concentration was obtained at a pH of 5.6, equivalent to a concentration of 0.74 g L<sup>-1</sup> (6.4 mM) of caproic acid, close to the upper toxicity limits reported for chain elongation (0.87 g L<sup>-1</sup>)<sup>11</sup>. Thus, the maximum concentration obtained in this study might be limited by toxic effects of the undissociated form of VFA. The presence of hydrogen in the system was detrimental to oxidation reactions due to product inhibition and, furthermore, it was steering elongation reactions between butyrate and acetate or CO<sub>2</sub> (Eq. 29 and 30) as also stated previously for propionate<sup>22</sup> or butyrate<sup>23</sup>.

MCFA such as valerate, caproate or iso-butyrate were only tested at high biomass concentrations. None of these compounds were elongated in the short term. The only reactions occurring were ethanol degradation, caproate oxidation to butyrate and, in the case of iso-butyrate, acetate was produced and further reduced to ethanol to a surplus concentration of 5 mM in comparison to the fed substrate. Therefore, using MCFA as direct substrate was not promoting further chain elongation. However, compounds such as caproate or heptanoate were produced when using shorter intermediates such as propionate or butyrate (Figure 2b). In this sense, initial toxicity concentration of MCFA was attributed to chain elongation

inhibition., as undissociated fatty acids have higher antimicrobial properties when the chain length increases <sup>24</sup> Taking into account the initial pH and concentration, 10 mM (1 g L<sup>-1</sup>) of valeric acid and 6 mM (0.7 g L<sup>-1</sup>) of caproic acid were present in C5 and C6 tests, respectively. Maximum concentrations of 3.7 mM (0.4 g L<sup>-1</sup>) of caproic acid and 0.5 mM (0.1 g L<sup>-1</sup>) of heptanoic acid were estimated at the end of tests C2 and C3. No caprylate was detected in any of the tests performed after 9 days of incubation.

### **Enrichments with different carboxylates as substrates for chain elongation**

HB tests were consecutively enriched by transferring each inoculum to fresh media. This enabled studying the microbial community adaptability to the target substrates (Figure S3, supporting information). Whereas a slight pH increase occurred during the first run, pH decreased by  $0.59 \pm 0.08$  units in the last incubation dropping to a minimum pH of 5. During enrichment, formate behaved differently to other carbon sources as total VFA production increased 12.6 times during the media transfers. Considering that hydrogen was easily generated by formate oxidation (Eq. 6; Figure S2, 23.8 mM), subsequent elongation of formate to ethanol or acetate (Eq. 7 and 8), acetate to butyrate (Eq. 13 & 16) and butyrate to caproate (Eq. 29 to 31) were enhanced by these reductive conditions. Traces of propionate generated by direct elongation with ethanol (Eq. 9) and subsequent elongation to valerate was also observed, accounting for 15% of odd carbon carboxylates in the final composition. Despite hydrogen production, acetate was still the main product formed at 60% of the final composition.

Acetate as a substrate slightly diminished the final total VFA production after enrichment, likely due to a decrease in inoculum degradation. Stable carboxylate production was observed in sequential batch tests fed with propionate as carbon source, although oxidation to acetate increased and further elongation to caproate decreased. The valerate composition was stable

around 50% in the subsequent transfers (Table S3). In butyrate batch experiments chain elongation to caproate switched to substrate oxidation to acetate as observed by the change in final VFA composition (Figure S3b, Table S3), probably due to a lower hydrogen presence<sup>23</sup>. Longer VFAs such as C5, C6 and iso-C4 were not suitable as raw materials for chain elongation in this study not even after adaptation (Figure S3). Valerate and caproate elongations with ethanol were supposed to be thermodynamically feasible (Eq. 35 & 39, Table 1), but no production nor substrate oxidation was detected in the enrichment tests. This fact was mostly due to undissociated acid toxicity since 20 mM of valeric acid (2.1 g L<sup>-1</sup>) and 20.7 mM of caproic acid (2.4 g L<sup>-1</sup>) were present at initial conditions. Iso-butyrate as substrate did not present inhibitory concentrations but the products were mainly based in oxidation products (i.e. acetate).

Carboxylates from C1 to C4 are feasible to use as substrates for chain elongation, although formate usage requires some acclimation. MCFA such as C5 and C6 and the iso-form of C4 are unviable for chain elongation when directly used as substrate at similar concentrations as the other VFA. Combinations of even carboxylate-ethanol lead to even MCFA. However, combinations of propionate-ethanol lead to a mixture of even and odd carbon carboxylate mixtures. Propionate increases the proportion of even VFA likely because of partial oxidation to acetyl-CoA which then drives the elongation. In an earlier study by Dennis et al.<sup>25</sup> it was hypothesized that valerate production from glycerol occurred through propanol formation which then elongated with acetate. Considering thermodynamics and the results obtained, valerate should be easily obtained from propionate under the presence of hydrogen and/or CO<sub>2</sub> (Eq. 22 & 23) or from the combination propionate-ethanol (Eq. 25).

## Community composition convergence in chain elongation

PCA analysis was performed at the end of each enrichment to relate the divergence of rRNA sequences from that of the initial inoculum with the chemical results. After 27 days with HB, all communities fed with acetate and different alcohols from methanol to butanol (control with water included) diverged from the inoculum and converged in a cluster with the exception of the test fed with butanol (AB) (Figure 3a). Richness values decreased from  $255 \pm 21$  (inoculum; I) to a range of  $137 \pm 19$  (acetate-methanol; AM) to  $224 \pm 4$  (acetate-butanol; AB) for all alcohol tests while Pielou evenness values remained stable with the exception of the ethanol test which decreased from  $0.66 \pm 0.01$  to 0.59. Even though the control test (acetate-water; AW) did not produce high amounts of VFAs (Figure 1 & S1), the community composition was similar to the one targeting chain elongation. Acetate-methanol (AM), with the lowest Richness value, showed the highest variability between biological replicates and thus slightly differing from the successful cluster. This variation was in line with the decrease in VFA production during enrichment (Figure S1) which might be attributed to methanol toxicity. Butanol was discarded as an electron donor due to its poor biochemical performance as a substrate (Figure S1) and its distribution in the PCA (AB, Figure 3a) was an outlier from the successful cluster.

Different clusters were observed when carboxylate combinations (from C2 to C6) were evaluated by PCA. Composition convergence was attributed to substrate instead of time adaptation as clusters for control samples (carboxylate-water; YW) diverged from the ones of the tests (carboxylate-ethanol; YE). Cluster convergence of tests performing chain elongation was already observed after 9 days inoculation. The Pielou evenness values slightly decreased from  $0.66 \pm 0.01$  to  $0.59 \pm 0.02$  for the tests successfully performing chain elongation (combinations of formate, acetate, propionate and butyrate-ethanol; FE, AE, PE, BE) and increased to 0.7 for non-successful substrates and controls. Richness values decreased to



values under  $200 \pm 14$  for the successful cluster while they remained similar to the inoculum at  $255 \pm 21$  for the rest of the tests. The substrate combination formate-ethanol (FE, Figure 3b) diverged from other clusters when correlating biochemical performance and community composition. This may be due to the need to initially convert formate to acetate in order to activate the chain elongation process (Eq 6; Figure 2 & S3). The rest of the tests which achieved chain elongation (combinations with acetate, propionate, butyrate-ethanol; AE, PE, BE) clustered together. This shows that similar communities allow chain elongation irrespective of the carboxylate used as substrate. The degradation of inoculum and substrate (i.e. acetate or propionate) to ethanol followed by a common chain elongation process situated the acetate and propionate control tests slightly outside the main cluster. Tests and controls for valerate and caproate combinations were grouped away from the common cluster for chain elongation. The high initial toxic concentrations present in the tests were unable to support growth and activity for chain elongation. Thus, their presence modified the microbial community composition in a different manner than suitable substrates.

Our results thus show a convergent evolution of bacterial communities different from the initial community present in the inoculum as long as substrate combinations led to chain elongation. Control experiments clustered differently from the inoculum and the successful cluster, demonstrating the influence of substrate rather than time evolution in the microbiome. The communities slightly differ from the successful cluster when substrate combinations require a preceding oxidation-reduction reaction or inoculum degradation to permit chain elongation in e.g. the control tests.

### **Microbial distribution in chain elongation**

*Clostridia* (45-57%) class consistently dominated when both ethanol and a viable carboxylate for chain elongation (C1 to C4) were supplied (one-way ANOVA,  $p < 0.001$ ). *Clostridia* only

reached 21-29 % of the total prokaryotic population when propanol and butanol were supplemented, thus not presenting significant differences between successful and unsuccessful chain elongation ( $p = 0.534$ ). Phylotypes (Phy) exhibiting 16S rRNA sequences identical to that of *Clostridium kluyveri* (Phy1, Figure S4), also detected earlier with acetate-ethanol combinations<sup>26</sup>, and Phy 11 (*Clostridium ljungdahlii/ragsdalei/autoethanogenum*; Figure S4) were enriched in all tests with carbon chain lengths greater than 2 (Figure 5) indicating these bacteria are key players for the success in chain elongation. Phy33, annotated as *Clostridiaceae I* in this study, was enriched along with Phy1 and 11 (Figure 5) and it is also associated (>99%, NCBI) with *Caloramator sp.* which produces ethanol, short chain fatty acids, carbon dioxide and hydrogen as end products of glucose fermentation<sup>27</sup>. The highest relative abundances of all these phylotypes were observed with ethanol/propanol and in propionate/butyrate. Phylotypes related to the *Ruminococcaceae* family were observed in acetate-ethanol tests (such as Phy31 or Phy51, annotated until genus level and related to *Oscillibacter sp.*, Figure 5). From the same family, Phy59 (annotated until genus level as *Clostridium IV*) was enriched in the ethanol combination with C2 to C4, but also with iso-butyrate (i-C4). Another organism also related to Cluster IV is *Clostridium sp.* BS-1. Isolates of BS-1 can produce caproate from fermentation of D-galacticol<sup>28</sup>. However, BS-1 enhances chain elongation to caproate with co-cultures of BS-7 or optimization of the medium<sup>29</sup>, thus suggestion that it was not the galacticol itself that elongated substrates but rather its intermediates. Members of *Ruminococcaceae* family were the key species found in a chain elongation study where corn fiber was used as substrate<sup>30</sup>.

The formate-ethanol combination was highly enriched with Phy12, related to the genus *Clostridium XIVb*, *Lachnospiraceae* family, and Phy21 (*Clostridium sensu stricto*), while methanol fed tests were enriched in Phy38 (*Eubacteriaceae*). Specific phylotypes for methanol or formate (C1 substrates) might be related to acetate or bigger compounds

formation. Finally, a single unclassified phylotype related to *Clostridiales* order (Phy20, Figure S4), was enriched using only alcohols different from ethanol. This particular phylotype has been also detected in anaerobic fermentation of xylose<sup>31</sup> and thermally hydrolysed activated sludge<sup>32</sup>, but it is also highly similar to the genus *Tissierella* (>95%, NCBI)<sup>33</sup>, an anaerobic non-sporulating cell that grows at mesophilic conditions with the presence of creatinine. It is considered a weaker fermenter than other *Firmicutes*, but produces acetic and other SCFA from peptone-yeast extract-glucose<sup>34</sup>. Its main function was postulated to be related to the oxidation of alcohol to SCFA.

Class-level *Bacteroidia* were present in all tests although differences were not statistically significant between the successful and unsuccessful tests (Figure 4,  $p = 0.359$ ). Enrichments related to *Bacteroidia* were only significant with single carbon substrates as methanol or formate (Phy61 and Phy104, family of *Porphyromonadaceae*, Figure 5). Specifically, both phylotypes have been previously detected in anaerobic digesters<sup>35</sup> and microbial fuel cells<sup>36</sup>, both systems dealing with complex substrates, but no correlation with bacterial function was postulated. Phylotypes related to *Bacteroidaceae* family (Phy8, annotated until genus level of *Bacteroides*), a common group encountered in mesophilic mixed culture fermentation<sup>37</sup>, were slightly enriched in all tests.

Species related to the class *Bacilli*, order *Bacillales*, have been detected previously when treating complex substrates<sup>38</sup>. In this study we detected the highest enrichment in *Bacillales* (Figure 5) with LB (26 to 68% of the total community), while the same phylotype was outcompeted by the order *Clostridiales* with HB. These microorganisms might speculatively be related to oxidation reactions.

*Erysipelotrichia* ( $p < 0.001$ ) was also present in most of the successful tests. Contrarily, classes *Negativicutes* ( $p = 0.002$ ; mainly from *Selenomonadales* order), *Bacilli* ( $p = 0.008$ )

and *Alphaproteobacteria* ( $p = 0.033$ ) dominated with substrates where chain elongation did not occur. *Microbacteriaceae* family (Phy4) was enriched in all test even with substrate not viable for chain elongation. This phylotype is related to *Pseudoclavibacter caeni* (100%, NCBI) which is related to the production of acids from sugars and assimilation of acetate, but does not have a positive response for hydrolytic enzymes<sup>39</sup>. *Rhodobacteraceae* family (Phy10) organisms were most enriched with C1 (methanol/formate) or C3 (propanol/propionate) substrates (Figure 5). Interestingly, an unclassified Bacteria (Phy7) was detected in all successful chain elongation tests. The sequence of this phylotype matches the one detected in studies of sludge alkaline fermentation<sup>40</sup>.

To conclude, we observed clear convergence and speciation of bacterial communities, as long as the carboxylate and alcohol substrate combination allowed chain elongation. These results also show the validity of our set-up in terms of replication, convergence of communities has earlier been observed as a trait of fermenters and digesters over time<sup>41</sup>. *Clostridia* of the *Clostridia sensu stricto* genus are enriched from undefined mixed microbial cultures when supplying any alcohol and carboxylate as substrates, with *Clostridium kluyveri*, the model MCFA producing species, the main detected organism in this study. The use of C1 substrates enhances the growth of other *Clostridia* predominantly members of the *Eubacteriaceae* family (methanol) or *Clostridium XIVb* (formate). However, the presence of other phylotypes related to *Bacteroidaceae*, *Microbacteriaceae* or unclassified bacteria suggests that other microorganism are directly or indirectly involved in the chain elongation process from mixed cultures.

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## SUPPORTING INFORMATION

Supporting information, available free of charge via the Internet at <http://pubs.acs.org>, contains: experimental set-up and chemical and molecular methodologies; excel containing sequences and identification of each phylotype from each sample; literature summary of the actual carboxylate productions (Table S1); Enrichment results for the acetate-alcohols and carboxylates-ethanol pairs are presented (Figures S1 and S3 and Tables S2 and S3, respectively). Control results for the carboxylate-ethanol combinations (Figure S2); and Neighbor-joining tree of the most enriched phylotypes (Figure S4).

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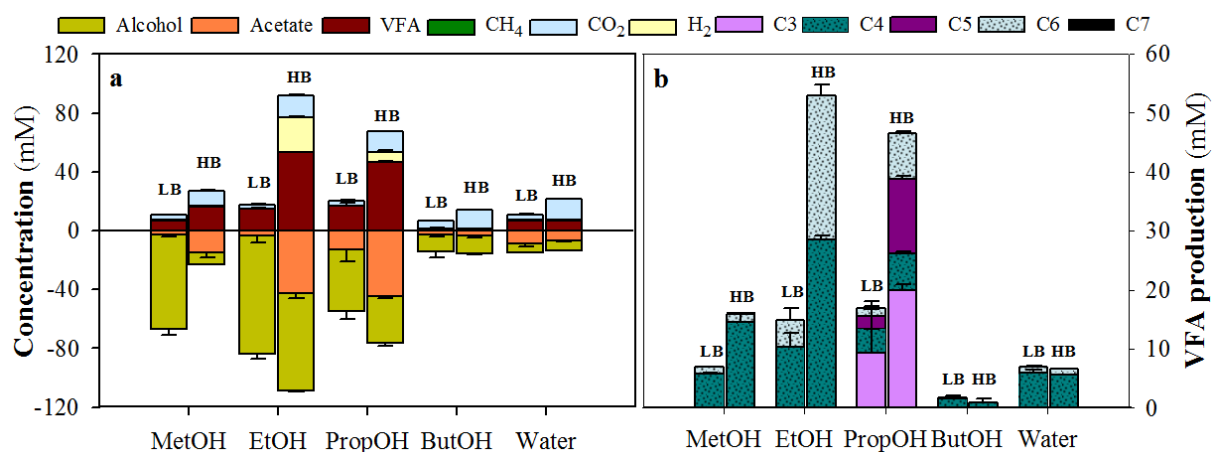
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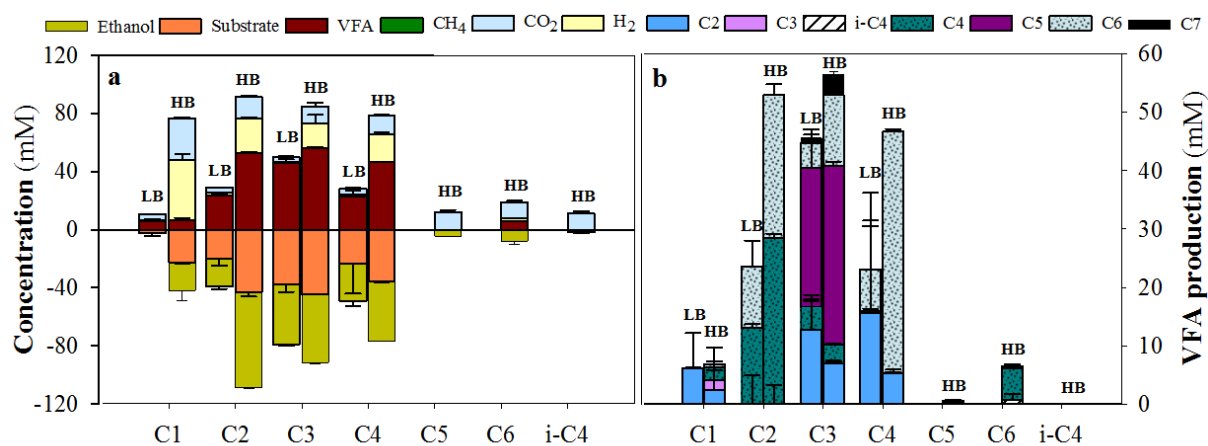


**Table 1.** Thermodynamic information of possible substrate combinations for oxidation reactions (grey stands for chain elongation reactions). Standard Gibbs energy change ( $\Delta G^0$ ) was calculated from the formation free Gibbs ( $G_f^0$ ) values of the compounds participating in the reaction <sup>15, 16</sup> (except Eq. 35 and 39 which were estimated by the Joback & Reid method <sup>17</sup>) and the stoichiometry of the reaction. Compensation by temperature ( $\Delta G^0_{35^\circ\text{C}}$ ) was calculated using the Gibbs-Helmholtz equation and correction at biological standard state (pH 7,  $\Delta G^0_{35^\circ\text{C}}$ ) according<sup>18</sup>.

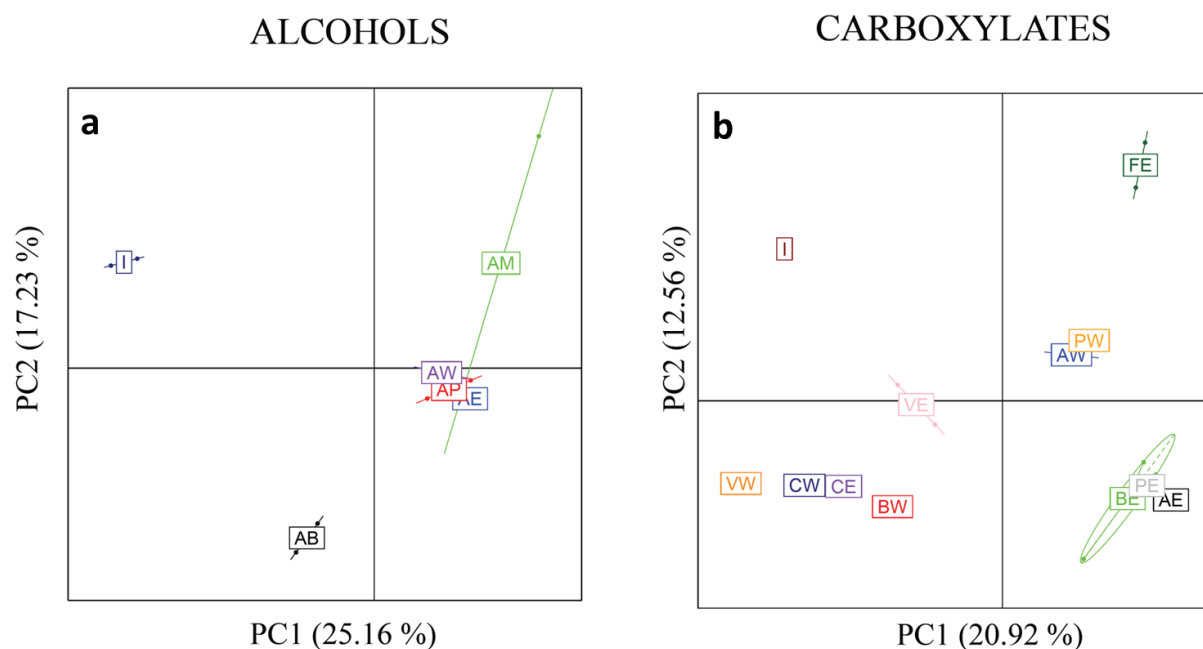
Substrate	Equation	#	$\Delta G^0$ (KJ mol <sup>-1</sup> )	$\Delta G^0_{35^\circ\text{C}}$ (KJ mol <sup>-1</sup> )	$\Delta G^0_{35^\circ\text{C}}$ (KJ mol <sup>-1</sup> )
<b>Methanol</b>	$\text{CH}_3\text{OH} + \text{H}_2\text{O} \rightarrow \text{HCOO}^- + \text{H}^+ + 2\text{H}_2$	1	61.5	60.2	20.3
<b>Ethanol</b>	$\text{CH}_3\text{CH}_2\text{OH} + \text{H}_2\text{O} \rightarrow \text{CH}_3\text{COO}^- + \text{H}^+ + 2\text{H}_2$	2	49.5	48.5	8.6
<b>Propanol</b>	$\text{CH}_3(\text{CH}_2)_2\text{OH} + \text{H}_2\text{O} \rightarrow \text{CH}_3\text{CH}_2\text{COO}^- + \text{H}^+ + 2\text{H}_2$	3	51.8	50.9	11.0
<b>Butanol</b>	$\text{CH}_3(\text{CH}_2)_3\text{OH} + \text{H}_2\text{O} \rightarrow \text{CH}_3(\text{CH}_2)_2\text{COO}^- + \text{H}^+ + 2\text{H}_2$	4	56.3	55.5	15.6
<b>CO<sub>2</sub></b>	$2\text{CO}_2 + 4\text{H}_2 \rightarrow \text{CH}_3\text{COO}^- + \text{H}^+ + 2\text{H}_2\text{O}$	5	-54.8	-47.6	<b>-87.5</b>
<b>Formate</b>	$\text{HCOO}^- + \text{H}^+ \rightarrow \text{CO}_2 + \text{H}_2$	6	-43.4	-45.9	<b>-6.0</b>
	$2\text{HCOO}^- + 5\text{H}_2 \rightarrow \text{CH}_3\text{CH}_2\text{OH} + 3\text{H}_2\text{O}$	7	-191.1	-188.0	<b>-187.9</b>
	$2\text{HCOO}^- + \text{H}^+ + 2\text{H}_2 \rightarrow \text{CH}_3\text{COO}^- + 2\text{H}_2\text{O}$	8	-141.6	-139.4	<b>-99.5</b>
+ ethanol	$\text{HCOO}^- + \text{CH}_3\text{CH}_2\text{OH} \rightarrow \text{CH}_3\text{CH}_2\text{COO}^- + \text{H}_2\text{O}$	9	-65.4	-64.4	<b>-64.4</b>
<b>Acetate</b>	$\text{CH}_3\text{COO}^- + 2\text{H}_2\text{O} \rightarrow \text{HCOO}^- + \text{CO}_2 + 3\text{H}_2$	10	98.2	93.5	93.5
	$\text{CH}_3\text{COO}^- + \text{H}^+ + 2\text{H}_2\text{O} \rightarrow 2\text{CO}_2 + 4\text{H}_2$	11	54.8	47.6	87.5
	$\text{CH}_3\text{COO}^- + \text{H}^+ + 2\text{H}_2 \rightarrow \text{CH}_3\text{CH}_2\text{OH} + \text{H}_2\text{O}$	12	-49.5	-48.5	<b>-8.6</b>
	$2\text{CH}_3\text{COO}^- + \text{H}^+ + 2\text{H}_2 \rightarrow \text{CH}_3(\text{CH}_2)_2\text{COO}^- + 2\text{H}_2\text{O}$	13	-88.0	-86.4	<b>-46.4</b>
	$3\text{CH}_3\text{COO}^- + 2\text{H}^+ + 4\text{H}_2 \rightarrow \text{CH}_3(\text{CH}_2)_4\text{COO}^- + 4\text{H}_2\text{O}$	14	-176.2	-172.9	<b>-93.1</b>
+ methanol	$\text{CH}_3\text{COO}^- + \text{CH}_3\text{OH} \rightarrow \text{CH}_3\text{CH}_2\text{COO}^- + \text{H}_2\text{O}$	15	-53.4	-52.7	<b>-52.7</b>
+ ethanol	$\text{CH}_3\text{COO}^- + \text{CH}_3\text{CH}_2\text{OH} \rightarrow \text{CH}_3(\text{CH}_2)_2\text{COO}^- + \text{H}_2\text{O}$	16	-38.5	-37.9	<b>-37.9</b>
	$2\text{CH}_3\text{COO}^- + \text{H}^+ + \text{CH}_3\text{CH}_2\text{OH} + 2\text{H}_2 \rightarrow \text{CH}_3(\text{CH}_2)_4\text{COO}^- + 3\text{H}_2\text{O}$	17	-126.7	-124.4	<b>-84.5</b>
+ propanol	$\text{CH}_3\text{COO}^- + \text{CH}_3\text{CH}_2\text{CH}_2\text{OH} \rightarrow \text{CH}_3(\text{CH}_2)_3\text{COO}^- + \text{H}_2\text{O}$	18	-36.2	-35.5	<b>-35.5</b>
+ butanol	$\text{CH}_3\text{COO}^- + \text{CH}_3(\text{CH}_2)_2\text{CH}_2\text{OH} \rightarrow \text{CH}_3(\text{CH}_2)_4\text{COO}^- + \text{H}_2\text{O}$	19	-31.9	-31.0	<b>-31.0</b>
<b>Propionate</b>	$\text{CH}_3\text{CH}_2\text{COO}^- + 2\text{H}_2\text{O} \rightarrow \text{CH}_3\text{COO}^- + \text{CO}_2 + 3\text{H}_2$	20	71.5	71.8	111.7
	$\text{CH}_3\text{CH}_2\text{COO}^- + \text{H}^+ + 2\text{H}_2 \rightarrow \text{CH}_3(\text{CH}_2)_2\text{OH} + \text{H}_2\text{O}$	21	-51.8	-50.9	<b>-11.0</b>
	$\text{CH}_3\text{CH}_2\text{COO}^- + 2\text{CO}_2 + 6\text{H}_2 \rightarrow \text{CH}_3(\text{CH}_2)_3\text{COO}^- + 4\text{H}_2\text{O}$	22	-142.8	-134.0	<b>-134.0</b>
+ acetate	$\text{CH}_3\text{CH}_2\text{COO}^- + \text{CH}_3\text{COOH} + 2\text{H}_2 \rightarrow \text{CH}_3(\text{CH}_2)_3\text{COO}^- + 2\text{H}_2\text{O}$	23	-88.0	-86.4	<b>-86.4</b>
+ ethanol	$\text{CH}_3\text{CH}_2\text{COO}^- + \text{CH}_3\text{CH}_2\text{OH} + 3\text{H}_2\text{O} \rightarrow 2\text{CH}_3\text{COO}^- + \text{CO}_2 + \text{H}^+ + 5\text{H}_2$	24	121.0	115.5	75.6
	$\text{CH}_3\text{CH}_2\text{COO}^- + \text{CH}_3\text{CH}_2\text{OH} \rightarrow \text{CH}_3(\text{CH}_2)_3\text{COO}^- + \text{H}_2\text{O}$	25	-30.2	-28.4	<b>-28.4</b>
<b>Butyrate</b>	$\text{CH}_3(\text{CH}_2)_2\text{COO}^- + 2\text{H}_2\text{O} \rightarrow 2\text{CH}_3\text{COO}^- + \text{H}^+ + 2\text{H}_2$	26	88.0	86.4	46.4
	$\text{CH}_3(\text{CH}_2)_2\text{COO}^- + 4\text{H}_2\text{O} \rightarrow \text{CH}_3\text{COO}^- + 2\text{CO}_2 + 6\text{H}_2$	27	142.8	134.0	134.0
	$2\text{CH}_3(\text{CH}_2)_2\text{COO}^- \rightarrow \text{CH}_3(\text{CH}_2)_4\text{COO}^- + \text{CH}_3\text{COO}^-$	28	-0.2	-0.2	<b>-0.2</b>
	$\text{CH}_3(\text{CH}_2)_2\text{COO}^- + 2\text{CO}_2 + 6\text{H}_2 \rightarrow \text{CH}_3(\text{CH}_2)_4\text{COO}^- + 4\text{H}_2\text{O}$	29	-143	-134.2	<b>-134.2</b>
+ acetate	$\text{CH}_3(\text{CH}_2)_2\text{COO}^- + \text{CH}_3\text{COO}^- + \text{H}^+ + 2\text{H}_2 \rightarrow \text{CH}_3(\text{CH}_2)_4\text{COO}^- + 2\text{H}_2\text{O}$	30	-88.2	-86.6	<b>-46.6</b>
+ ethanol	$\text{CH}_3(\text{CH}_2)_2\text{COO}^- + \text{CH}_3\text{CH}_2\text{OH} \rightarrow \text{CH}_3(\text{CH}_2)_4\text{COO}^- + \text{H}_2\text{O}$	31	-38.7	-38.1	<b>-38.0</b>
<b>Valerate</b>	$\text{CH}_3(\text{CH}_2)_3\text{COO}^- + \text{H}_2\text{O} \rightarrow \text{CH}_3\text{CH}_2\text{COO}^- + \text{CH}_3\text{CH}_2\text{OH}$	32	38.5	37.9	37.9
	$\text{CH}_3(\text{CH}_2)_3\text{COO}^- + 2\text{H}_2\text{O} \rightarrow \text{CH}_3\text{COO}^- + \text{CH}_3\text{CH}_2\text{COO}^- + \text{H}^+ + 2\text{H}_2$	33	88.0	86.4	46.5
	$\text{CH}_3(\text{CH}_2)_3\text{COO}^- + 4\text{H}_2\text{O} \rightarrow 2\text{CH}_3\text{COO}^- + \text{CO}_2 + \text{H}^+ + 5\text{H}_2$	34	159.5	153.4	153.4
+ ethanol	$\text{CH}_3(\text{CH}_2)_3\text{COO}^- + \text{CH}_3\text{CH}_2\text{OH} \rightarrow \text{CH}_3(\text{CH}_2)_5\text{COO}^- + \text{H}_2\text{O}^*$	35	-38.5	-38.1	<b>-78.0</b>
<b>Caproate</b>	$\text{CH}_3(\text{CH}_2)_4\text{COO}^- + \text{H}_2\text{O} \rightarrow \text{CH}_3(\text{CH}_2)_2\text{COO}^- + \text{CH}_3\text{CH}_2\text{OH}$	36	38.7	38.1	38.0
	$\text{CH}_3(\text{CH}_2)_4\text{COO}^- + 4\text{H}_2\text{O} \rightarrow 3\text{CH}_3\text{COO}^- + \text{H}^+ + 4\text{H}_2$	37	176.2	172.9	172.9
	$\text{CH}_3(\text{CH}_2)_4\text{COO}^- + 2\text{H}_2\text{O} \rightarrow \text{CH}_3(\text{CH}_2)_2\text{COO}^- + \text{CH}_3\text{COO}^- + \text{H}^+ + 2\text{H}_2$	38	88.2	86.6	46.6
+ ethanol	$\text{CH}_3(\text{CH}_2)_4\text{COO}^- + \text{CH}_3\text{CH}_2\text{OH} \rightarrow \text{CH}_3(\text{CH}_2)_6\text{COO}^- + \text{H}_2\text{O}^*$	39	-38.5	-38.1	<b>-78.1</b>



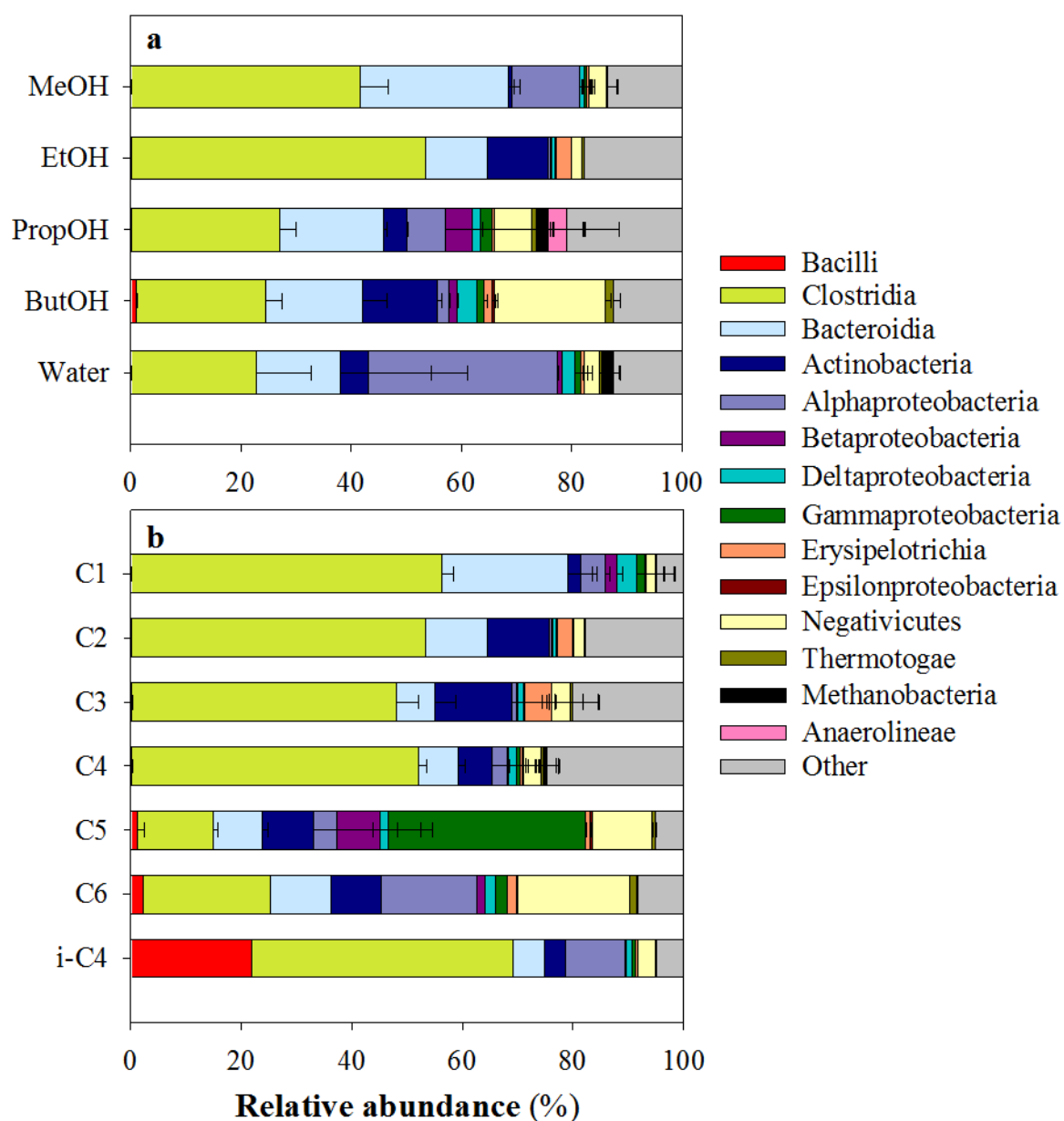
**Figure 1.** Substrate consumption (negative values) and product formation (positive values) (a) and VFA product distribution (b) after 9 days incubation with different alcohols as electron donor for Low biomass (LB) and High Biomass (HB) tests. VFA label includes all carboxylates specified in plot (b).



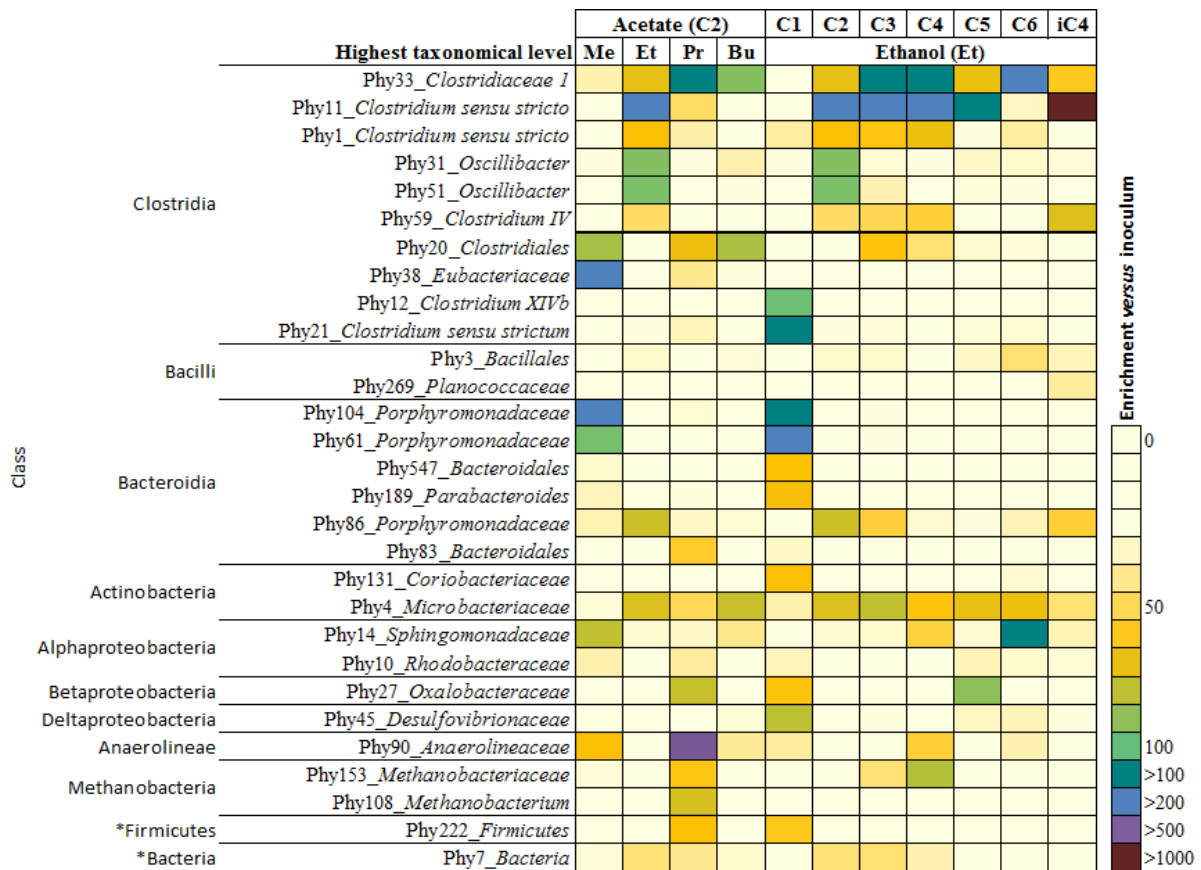
**Figure 2.** Substrates (negative values) and products (positive values) compounds (a) and VFA product distribution (b) after 9 days incubation with different carboxylates as electron acceptor for Low biomass (LB) and High Biomass (HB) tests. VFA label includes all carboxylates specified in plot (b).



**Figure 3.** Principal component analysis of the enriched mixed communities (HB, 27 days) showing the mixed microbial composition difference between samples in two dimensions (PC1, PC2) according to the effect of substrate mix composition and VFA production profiles. a) Alcohol tests being I: inoculum and AX substrate combination with A as acetate and X for methanol (M), ethanol (E), propanol (P), butanol (B) and water (W). b) Carboxylate tests being I: inoculum and YE or YW substrate combination with Y for formate (F), acetate (A), propionate (P), butyrate (B), valerate (V), or caproate (C) and E for ethanol or W for water. Dots represent the replicates for each experiment while the ellipse indicates two thirds of the replicates. Samples only seen by a box (label) correspond to results of close replicates.



**Figure 4.** Class-level relative abundance of the alcohol (a) and carboxylate (b) tests after enrichment transfers (high biomass, 27 days). Vertical axis shows the changing alcohol (combined with acetate) or carboxylate (combined with ethanol) used as substrate in each tests.



**Figure 5.** Heat map of the most enriched Phy compared to the inoculum (end point/inoculum) for all substrate combination tests after 27 days.